United States Department of Agriculture

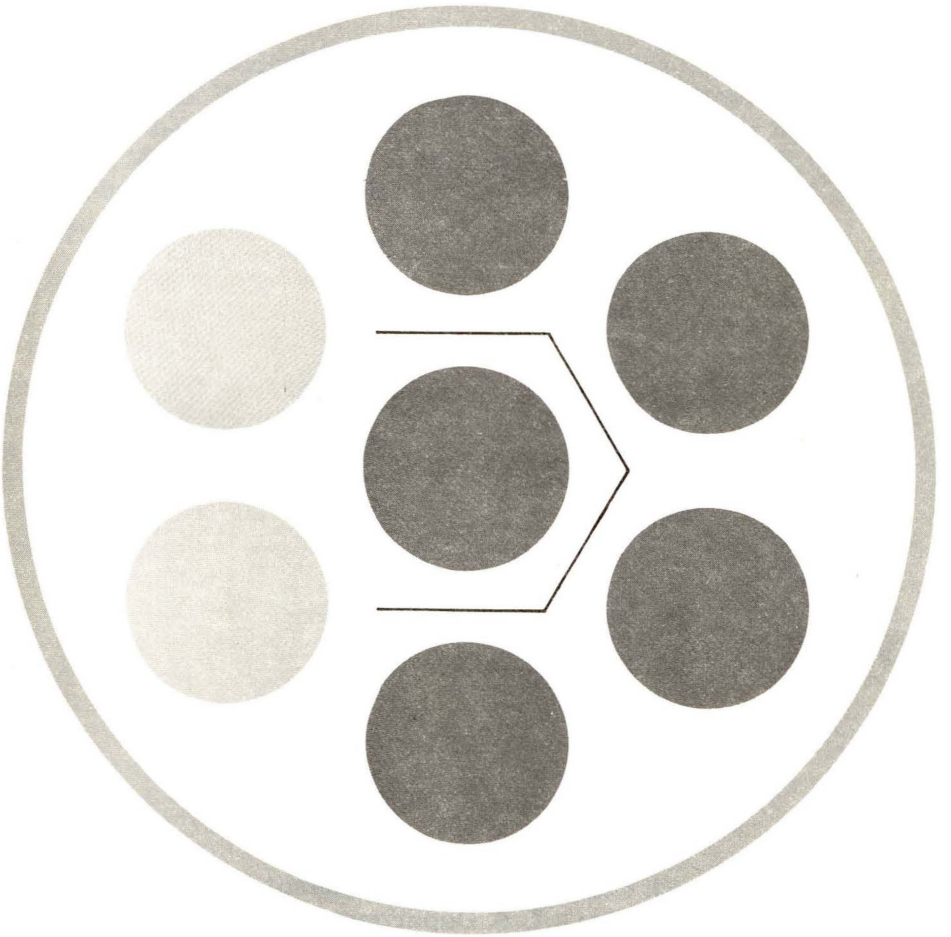
Forest Service

North Central Forest Experiment Station

General Technical Report **NC-87**

A Serological Procedure for Identifying Strains of Gremmeniella abietina

Darroll D. Skilling and Mariann Kienzler



North Central Forest Experiment Station
Forest Service—U.S. Department of Agriculture
1992 Folwell Avenue
St. Paul, Minnesota 55108
Manuscript approved for publication February 23, 1983
December 1983

PREFACE

As stated by Ball *et al.* (1974), "all serological tests for plant viruses (in our case, fungi) are modifications of the precipitin test in which a proteinaceous substance of a small molecular size combines with its specific antibody to produce an aggregate or precipitate that scatters enough light to be visible". Numerous serological techniques have been developed by investigators with various modifications. This manual uses the Ouchterlony double diffusion method. Other tests may be equally or more valuable depending on the situation.

Numerous individuals contributed in various ways to the development of this manual. We would like to acknowledge the assistance of Denise Dupont in the early development of our serology techniques. Dr. Thomas Nicholls, Michael Ostry, Dr. Mitchel Miller, and Professor Ernest Banttari also helped in the manual development and reviewed the final manuscript.

CONTENTS

	Page
Introduction to the serological procedure	1
Test material	2
Handling the test organism and producing antigen	2
Handling procedures for quarantined material	2
Isolating *G. abietina* from field samples	2
Growing *G. abietina* in liquid culture	3
Extracting soluble protein "antigens" from mycelial cultures	4
Appendix	5
Preparing antigens for the double diffusion test	6
Establishing a standard curve for protein level analysis	6
Preparing antigens for optical density test	6
Calculating concentrations of antigen protein	7
Diluting and storing antigens	8
Cross-absorption of antigens and antibodies	8
Appendix	9
Producing antiserum	10
Producing antigens for rabbit injection	10
Injecting antigens	10
Harvesting and storing antiserum	10
Appendix	11
Ouchterlony double diffusion method	12
Diffusion plates and design	12
Preparing double diffusion plates	12
The double diffusion plate design	12
Filling the double diffusion plate wells	12
Interpreting plates	13
Analyzing precipitin bands	13
Glossary	15
Literature cited	15

A SEROLOGICAL PROCEDURE FOR IDENTIFYING STRAINS OF *GREMMENIELLA ABIETINA*

Darroll D. Skilling, *Principal Plant Pathologist,*
and **Mariann Kienzler,** *Microbiologist*

INTRODUCTION TO THE SEROLOGICAL PROCEDURE

In 1977 Dorworth *et al.* determined that a newly discovered and highly virulent isolate of *Gremmeniella abietina* (Lagerb.) Morelet was present in northern New York State. Recent research has determined that this isolate is potentially one of the most serious diseases of conifers in North America. It has the ability to cause heavy losses in most pine species from seedlings to saw log trees. It also infects other conifers including spruce, fir, larch, Douglas-fir, and hemlock. The isolate of *G. abietina* found in New York was immunologically identical to isolates of this fungus from Europe but was different from all isolates of *G. abietina* found throughout the rest of North America. This isolate was called the European strain to distinguish it from the more common North American strain found in other areas of North America. Because the damage from the European strain is much more severe than that caused by the North American isolate and because the European strain is limited in its North American distribution, it has been placed under State and Federal quarantines in both the United States and Canada. In most cases this quarantine is specific to the European strain. As a result, it is necessary to identify the strain in newly infected areas before quarantine regulations can be established and enforced.

The objective of this manual is to describe a standard laboratory method developed to accurately identify strains of *G. abietina*. Several differences between the strains have been found and used to try to identify strains of *G. abietina*. These include growth rate, texture, and color of mycelial cultures; height of infection in host trees; and number of septations found in conidia. All of these characteristics have some value for identifying strains but none are completely reliable under all conditions. The only procedure that gives consistently reliable results is serology using the agar double diffusion test. The original serological technique for identifying strains of *G. abietina* was developed by Dorworth and Krywienczyk in 1975. This system has been adapted and refined at the North Central Forest Experiment Station and is presented here so that other forest pathology laboratories can use the technique to identify strains of *G. abietina* and, perhaps, strains in other fungus species.

The value of serological techniques and the antigen-antibody reactions is their specificity. An antibody will only combine with an antigen that contains amino acid sequences identical to that causing its formation in an animal. The production of virus antisera has been known for more than 50 years. In the 1940's the introduction of agar diffusion techniques made possible a procedure for determining not only the quantity but also the kinds of antigenic components within an antigen-antibody system (Ball 1974). In forest pathology, the use of serological techniques is more recent. Indications are that the serological techniques now used by plant virologists are adaptable with slight modifications to forest pathology fungi. The system outlined here is not limited to one species of fungi. With slight modification it appears that it will also identify strains within other species of fungi that may have developed slight genetic variations. Other serological methods can be used for this purpose. Fluorescent immunoassay and immuno-electrophoresis can, in many cases, give equal or better results. When possible investigators should try several tests to find the most useful and accurate method to solve a serological problem.

TEST MATERIAL

During 1980 and 1981 researchers at the North Central Forest Experiment Station screened 112 field isolates of *G. abietina* using the Ouchterlony double diffusion test. A total of 58 isolates were received from Canada; 34 from New York, Maine, and Vermont; and 20 from the Lake States. All of the isolates collected in the Lake States were identified to be the North American strain. Of the 92 isolates from Canada and the Northeast, 83 were the European strain. The isolates from Canada were also tested at the Great Lakes Forest Research Centre at Sault Ste. Marie, Ontario, as a check on the accuracy of the serological system. In more than 90 percent of these tests the results at the two laboratories were identical. In most cases any variation in the results of the two tests were either because the isolate was not *G. abietina* or because the isolate gave a positive reaction to both North American and European antiserum, which indicates that it had some characteristics of both strains. A confidence level of more than 90 percent in the area of race determination of higher fungi actually exceeds the rate of success one might expect in classical morphological determination of some problem fungi at the species level. Although the system is not as yet 100 percent accurate, identification errors are reduced as the procedure is improved. Although based on science, the serological process still has some aspects that can best be described as art. Until all the variables in this system are fully understood, some questions in results will continue. To avoid errors in strain identification, any deviation in readings between serology plates should be evaluated and discarded and the procedure should be rerun until results are consistent. With these precautions the accuracy rate should approach 100 percent.

This manual outlines the procedures used to handle field samples of *G. abietina* from the time they arrive at the laboratory until their strain is serologically identified.

HANDLING THE TEST ORGANISM AND PRODUCING ANTIGEN

Handling Quarantined Material

The European strain of *G. abietina* is under State and Federal quarantines so it is necessary to take additional safeguards when working with this organism. If the infected plant material is to be moved between States, it is necessary to obtain an interstate shipping permit (Plant Pest Quarantine Form 549) from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Room 630, Federal Building, Hyattsville, MD 20782. The permit also requires the signed approval of the official responsible for quarantine regulations for the State receiving the sample. Each sample shipment must have a separate permit attached.

The primary hazard in working with an organism such as *G. abietina* is that the fungus may be spread into healthy areas. To avoid this, strict laboratory security procedures must be followed. All rooms in which the fungus is handled should be clearly posted with signs informing workers and the general public of the hazards involved. Any materials used in those rooms must be autoclaved when they are removed. In addition to autoclaving infected plant material and cultures, all material in waste containers should be autoclaved. Any liquid used in these areas in conjunction with isolations or serology should be collected in buckets for autoclaving before going into the drain system. Janitorial services are best handled by laboratory personnel with separate equipment. Floor debris should also be autoclaved. All fungus cultures should be kept in separate incubators with quarantine labels prominently displayed. Where possible, incubators should be kept locked as a further security measure.

Isolating *G. abietina* from Field Samples

Successful isolation of *G. abietina* from field samples depends on the care used in handling the sample prior to its arrival in the laboratory. *G. abietina* does not remain viable under high temperatures so sample material must be kept cool during shipment and storage. Samples collected during May and June will usually yield a high percentage of pure cultures of *G. abietina*. This is due in part to the presence of the fungus fruiting bodies during this period. Isolation from infected plant material during July and August is usually more difficult. It appears that *G. abietina* is not actively growing during this period and saprophytic fungi frequently are the dominant organisms present in the dead plant tissue. Aseptic removal of conidia or ascospores from these fruiting bodies will usually give a pure culture of the fungus in about 7 days when grown at 5°C (fig. 1). If fruiting bodies are not present, it is possible to isolate the fungus directly from newly infected plant tissue using standard pathological isolation procedures. Branches showing the characteristic green stain of *G. abietina* in the cambial zone are a good source of

Figure 1.—*Conidia of* G. abietina *on branch.*

isolation material. Wood chips containing *G. abietina* may be surface sterilized with a 1:10 hypochlorite solution (household bleach and water) for 1 minute, although this procedure is not always necessary (fig. 2). Although *G. abietina* will grow on standard malt agar media, it grows more rapidly if Campbell's V-8 juice[1] is substituted for 20 percent of the water (see Appendix). The fungus grows slowly at 25°C and will usually be overgrown by other fungi that invade tissue killed by *G. abietina* and that grow well at warmer incubation temperatures. To avoid this problem, the original isolations should be incubated at 5°C. At 5°, *G. abietina* is able to grow moderately while most of the commonly isolated saprophytic fungi are not. After a pure mycelial culture is obtained from the original transfer, it should be incubated at 15-17°C for optimum mycelial growth. Cultures grow well in the dark but should be grown under fluorescent light if asexual fruiting is desired.

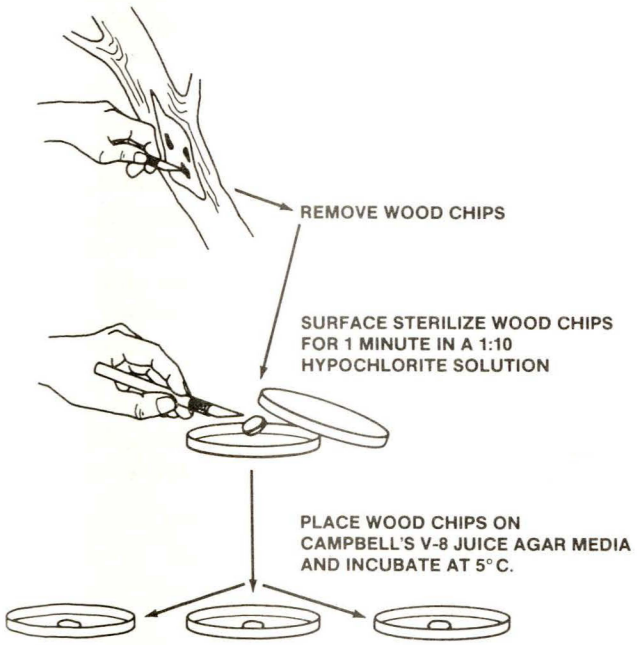

REMOVE WOOD CHIPS
SURFACE STERILIZE WOOD CHIPS FOR 1 MINUTE IN A 1:10 HYPOCHLORITE SOLUTION
PLACE WOOD CHIPS ON CAMPBELL'S V-8 JUICE AGAR MEDIA AND INCUBATE AT 5° C.

Figure 2.—*Method of isolating* G. abietina *from branches showing the characteristic green stain in the cambial zone.*

Growing *G. abietina* in Liquid Culture

Objective

Produce sufficient mycelium for homogenizing and extracting soluble antigens for protein determination.

G. abietina grows well in a liquid media consisting of Campbell's V-8 vegetable juice and glucose. Vegetable juice should be filtered to remove pulp before adding to media.

Materials

Several 10-inch sheets Miracloth (see Appendix)
420 ml/sample Campbell's V-8 juice (see Appendix)
8–foam stoppers/sample
8–100 ml beakers/sample
1–Buchner funnel 14 cm
8–500 ml Erlenmeyer flasks/sample
Several sheets GF-A filter paper 12.5 cm
Several sheets glass microfiber paper
20 g–D. glucose anhydrous (granular)
2–graduated cylinders
1–siphon hose
1–1,000 ml side-arm Erlenmeyer flask
1–500 ml plastic bottle/sample
1–2 liter flask

Procedure

A. Centrifuge 420 ml of V-8 juice per sample at 9,000 rpm for 10 minutes. Line the bottom of

[1]*The use of trade and company names is for the benefit of the reader; such use does not constitute an official endorsement or approval of any service or product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.*

the Buchner funnel with 1 layer of Miracloth and insert it into a 1,000 ml side-arm flask with vacuum line attached. Slowly pour the supernatant through the funnel.

B. Fill plastic bottles ⅔ full with filtrate and freeze to get additional separation of the vegetable pulp.

C. After freezing for about 8 hours, allow the plastic bottles with the filtrate to thaw undisturbed. During this time additional pulp will settle to the bottom of the bottles. After the pulp has settled, siphon off the supernatant into a 2-liter flask. Filter supernatant through a Buchner funnel under vacuum with a sheet of GF/A glass microfiber (see Appendix) paper on the bottom of the Buchner funnel.

D. To prepare growth media add 20 g glucose and 1,800 ml distilled water to 200 ml of V-8 filtrate. This is sufficient media for 8 flasks of one culture.

E. Dispense 250 ml of media into each of 8-500 ml Erlenmeyer flasks. Seal with with foam plugs and cover the flask necks with 100 ml beakers.

F. Autoclave immediately for 20 minutes at 15 psi to prevent bacterial growth in the media. If bacterial contaminates are present, the media will appear cloudy and should not be used.

G. After cooling, inoculate the flasks with mycelial plugs of the test organism taken from the margin of the mycelial colony. We use 4 or 5, 5mm^2 plugs per flask.

H. Incubate the inoculated flasks in the dark at 17°C for 21 days. Shake flasks daily to aerate the mycelium and to break mycelial mats into smaller fragments for easier extraction.

Extracting Soluble Protein "antigens" from Mycelial Cultures

Objective

Remove soluble proteins from fungus mycelium grown in liquid culture.

This process involves separating mycelium from liquid media followed by washing and homogenizing mycelium in a cell homogenizer to break down the mycelial cell structure. The resulting soluble proteins (antigens) are then filtered from the homogenized mycelium.

Materials

A. For removing mycelium from liquid media:
Several 10-inch sheets Miracloth (see Appendix)
1–glass stirring rod/sample
1 pair–surgical gloves/sample
1–1,000 ml beaker/sample
Parafilm (see Appendix)
1–bucket for autoclaving all waste liquid
Ice chest and ice
1–150 ml beaker/sample
Triple-distilled H_2O

B. For homogenizing and centrifuging:
Braun MSK cell homogenizer (see Appendix) or other suitable homogenizer
1 or 2–60 ml/homogenizer flasks/sample[2]
1–glass rod/sample
2 liters–phosphate buffer 6.9 pH (see Appendix)
CO_2 cylinder for homogenizer
1–250 ml plastic centrifuge bottle/sample
Refrigerated centrifuge
Ice chest and ice
Acetone (for rinsing)
Glass beads 1.00–1.05 mm (see Appendix)

C. For filtering:
1–500 ml side-arm flask/sample
1–prefilter, No. AP2504700 (see Appendix)/sample
1–Millipore filter assembly (see Appendix)/sample
1–250 ml flask/sample
Parafilm (see Appendix)
Ice chest and ice
Phosphate buffer 6.9 pH (see Appendix)
Triple-distilled water

Procedure

During the entire process of extracting soluble protein antigens, be careful to avoid contaminating mycelium with foreign proteins. All equipment must be clean and rinsed with triple-distilled water. Surgical gloves should be worn to avoid skin contact with the mycelium. Contamination can be a problem when several mycelial isolates are being extracted at the same time. Cross contamination with other samples will completely destroy the accuracy of the final serological reading. **Change gloves, beakers, and stirring rod after working with each sample.**

A. Filter liquid media and mycelium through 1 layer of Miracloth into a beaker. Pour (excess) fluid from beaker into a quarantine bucket to be autoclaved (fig. 3).

B. After filtering all 8 flasks of mycelium, carefully squeeze excess liquid from mycelium by twisting Miracloth.

[2]*Millipore assemblies and homogenizing flasks can be reused when washed 3 times with distilled water, twice with acetone, and then air-dried.*

Figure 3.—*Filter liquid media and mycelium through Miracloth and then pour excess fluid into a quarantine bucket.*

C. Rinse mycelium with triple-distilled water until rinse water remains clear to remove traces of growth media from mycelium. Stir and break up clumps of mycelium with a glass rod so that entire fungus mass is thoroughly rinsed.

D. Between rinsings and after last rinse, squeeze the Miracloth to remove the excess fluid from mycelium. All liquid must go into quarantine bucket.

E. Place mycelial pellet into a prelabeled beaker, cover with Parafilm and place on ice until it can be *homogenized*. **Do Not Freeze.**

F. Break up mycelial pellet with glass rod and add a small amount of phosphate buffer (pH 6.9) to moisten pellet.

G. Fill a 60 ml cell homogenizer flask one-half to two-thirds full of mycelium. Add 1 cm of glass beads. Add phosphate buffer until beads are just covered.

H. Place flask in homogenizer for 40 seconds or until mycelium is completely homogenized. Adjust CO_2 valve on homogenizer to keep mycelium cold but not frozen. Repeat operation if entire mycelial pellet has not been homogenized. Keep homogenized mycelium cold for the rest of the process by placing beakers on crushed ice.

I. Place homogenized mycelium and glass beads in a plastic centrifuging bottle. Residue left in flask should be rinsed out with distilled water into a quarantine bucket. Keep bottle with homogenized mycelium on ice until centrifuged. Centrifuge as soon as possible at 9,000 RPM for 10 minutes at 0°C.

J. **Use clean glassware for each sample. Do not cross-contaminate. Autoclave all equipment and liquid used to kill any viable mycelium that may remain.**

K. Place a Millipore assembly consisting of a prefilter No. AP2504700 over a screen (see Appendix) in a 500 ml side-arm Erlenmeyer flask attached to a vacuum line. After second centrifugation, filter the supernatant through the assembly. Pour liquid out slowly leaving glass beads and sludge in plastic bottle.

L. Rinse beads and sludge from plastic bottle so that bottle can be cleaned and reused.

M. Transfer filtrate (antigen) into 250 ml Erlenmeyer flasks, cover with Parafilm, and chill on ice. The antigens remain viable for up to 24 hours if kept cool (10°C). We strongly suggest that the protein level analysis be completed soon after harvesting and homogenizing so that the levels of nitrogen are at their optimum. This procedure is explained in detail in the following section.

Appendix

A. Isolating *G. abietina* from field samples

V-8 media for 4 samples

1. 45 g malt agar
2. 200 ml V-8 juice (unfiltered)
3. 800 ml distilled water

Mix all ingredients in a 1500 ml flask. Cover with foam stopper and 100 ml beaker. Autoclave for 20 minutes at 15 psi. In an aseptic pouring area fill petri dishes one half full with media. Store in 5° incubator until needed.

B. Growing *G. abietina* in liquid culture and extracting soluble proteins.

Materials and sources of supply:

Parafilm–Dixie marathon, Greenwich, CT 06830

Miracloth–Calbiochem, P.O. Box 17, Elk Grove, IL

V-8–Campbell product

GS-A-12.5 cm glass filter paper–Whatman Lab. Products, Inc.,Clifton, NJ

Braun MSK cell homogenizer and flasks–B. Braun Instruments, 807 Grandview Dr. South, San Francisco, CA 94080

Glass beads–size 1.00–1.05 mm–B. Braun Instruments

Millipore assembly–Millipore Corp., Bedford, MA 01730
Phosphate buffer
1. 1 liter distilled water
2. 13.61 g KH_2PO_4
3. 26.81 g NaH_2PO_4
4. 34.0 g NaCl
5. NaOH–or Hcl
- Combine first 4 ingredients
- Read pH on pH meter
- Adjust pH to 6.9 by adding NaOH or Hcl

PREPARING ANTIGENS FOR DOUBLE DIFFUSION TEST

Establishing a Standard Curve for Protein Level Analysis

Objective

Equalize the protein levels of culture samples necessary for the double diffusion test. This standardizing is accomplished by using a modified Lowry Test(s) that establishes a standard curve using optical density and parts per million of protein of the known protein solution. A standard curve is set up each time the protein analysis is run because there are influencing variables, i.e., exact amounts of reagents added to the unknowns, temperature changes, and age of chemicals used.

Materials

17–16 ml test tubes for standards and blanks
2–test tube racks
1–50 ml volumetric flask
3–100 ml beakers
1–250 ml graduated cylinder
1–500 ml repeating pipette calibrated in 5 ml
1–10 ml graduated cylinder
Known protein solution (see Appendix)
Copper reagent (see Appendix)
Folin (see Appendix)
Triple-distilled wate
Balance
Spectrophotometer
Weighing paper
Magnetic stirrer
Test tube mixer
Disposable pipettes and dispenser
1 pipette for water
1 pipette for known protein solution
1 pipette for each antigen sample
Ice–(antigens must be kept at about 5°C)

Procedure

A. Arrange 6 sets of 16 ml test tubes in one of the 2 test tube racks in the following manner:
1. 2–16 ml tubes in row 1 (these are used to calibrate the spectrophotometer)
2. 3–16 ml tubes in rows 2, 3, 4, 5, and 6.
B. Weigh out 0.050 g of the Bovine Albumin (see Appendix). (This yields 1,000 ppm protein when added to 50 ml H_20).
C. Add the 0.050 g albumin to a volumetric flask and slowly mix in the 50 ml of triple distilled H_20. Mix the solution carefully because excessive bubbling will denature the proteins.
D. Set up a test series by pipetting the diluted Bovine Albumin into the test tubes.
1 ml = 1,000 ppm protein–row 2 (all 3 tubes)
0.8 ml = 800 ppm protein–row 3 (all 3 tubes)
0.6 ml = 600 ppm protein–row 4 (all 3 tubes)
0.4 ml = 400 ppm protein–row 5 (all 3 tubes)
0.2 ml = 200 ppm protein–row 6 (all 3 tubes)
E. Pipette the appropriate amount of triple-distilled H_20 to each set of test tubes to equal 1 ml (refer to table 1).
F. Following the establishment of the known protein series, prepare the antigens for the optical density test by adding the copper reagent, Folin (see Appendix), and water as described in the next section.

Preparing Antigens for Optical Density Test

Procedure

A. Set up three, 16 ml test tubes per fungus sample (to allow for additional readings and accuracy). Add 0.1 ml antigen and 0.9 ml triple-distilled water to each test tube. Use separate pipettes for each antigen sample. Label each sample.
B. Add 5 ml of the copper reagent (see Appendix) (using the repeating pipette) to all known protein

Table 1.—*Amounts of the known protein and triple-distilled water needed to obtain desired concentrations for the standard curve*

Tubes needed (number)	Desired amount known protein	Amount known protein added per tube	Triple-distilled H_2O added per tube
	ppm	*ml*	*ml*
2[1]	0	0	1
3	1,000	1	0
3	800	0.8	0.2
3	600	0.6	0.4
3	400	0.4	0.6
3	200	0.2	0.8

[1]Used to calibrate spectrophotometer.

Table 2.—*Test solutions in ml required to produce protein levels for preparing standard curve and antigens for optical density test*

Test solution	Desired protein level (ppm)				
	1,000	800	600	400	200
Protein solution	1	0.8	0.6	0.4	0.2
Distilled H_2O	0	0.2	0.4	0.6	0.8
Copper reagent	5	5	5	5	5
Folin reagent	0.5	0.5	0.5	0.5	0.5

standards and antigen samples (shown in table 2). Mix all tubes using a vibrating mixer.

C. Wait 10 minutes.

D. Pipette 0.5 ml of Folin reagent (see Appendix) into each standard and sample test tube (as shown in table 2).

E. Mix on vibrating mixer.

F. Wait 30 minutes. (The spectrophotometer should be turned on and calibrated according to directions at this time.)

G. Measure the known protein dilutions with the spectrophotometer to determine the optical density readings for each protein level. Record the optical density readings (as shown in table 3).

H. Run the antigen samples through the spectrophotometer to determine their optical density. Record these readings (as shown in table 4).

Table 3.—*Typical optical density readings for different protein levels of known solutions and antigen samples*

Desired protein (ppm)	Actual protein of known solution	Optical density[1] expressed in wavelength		
		A	B	C
	ppm			
1,000	1,000	0.10	0.10	0.10
800	800	.80	.80	.80
600	600	.60	.60	.60
400	400	.40	.40	.40
200	200	.20	.20	.20
	Undetermined			
Antigen sample	protein ppm			
A	—	.40	.42	.40
B	—	.67	.67	.68
C	—	.57	.58	.58

[1]The figures shown in this table are examples of the measurement of color intensity created by reagent and organic nitrogen. They are not to be used in calculations.

Table 4.—*Using the optical density wavelength of the unknowns to establish ppm of protein*

Sample	Optical density				Graph reading
	1	2	3	Average	
A	0.40	0.42	0.41	0.41	460
B	.50	.50	.51	.50	580
C	.65	.65	.66	.65	710

Calculating Concentrations of Antigen Protein

Procedure

A. After determining the optical density for the known protein solutions and the antigen samples, plot the optical densities for the known proteins (see fig. 4). Then, plot the average optical density of the three samples for each antigen.

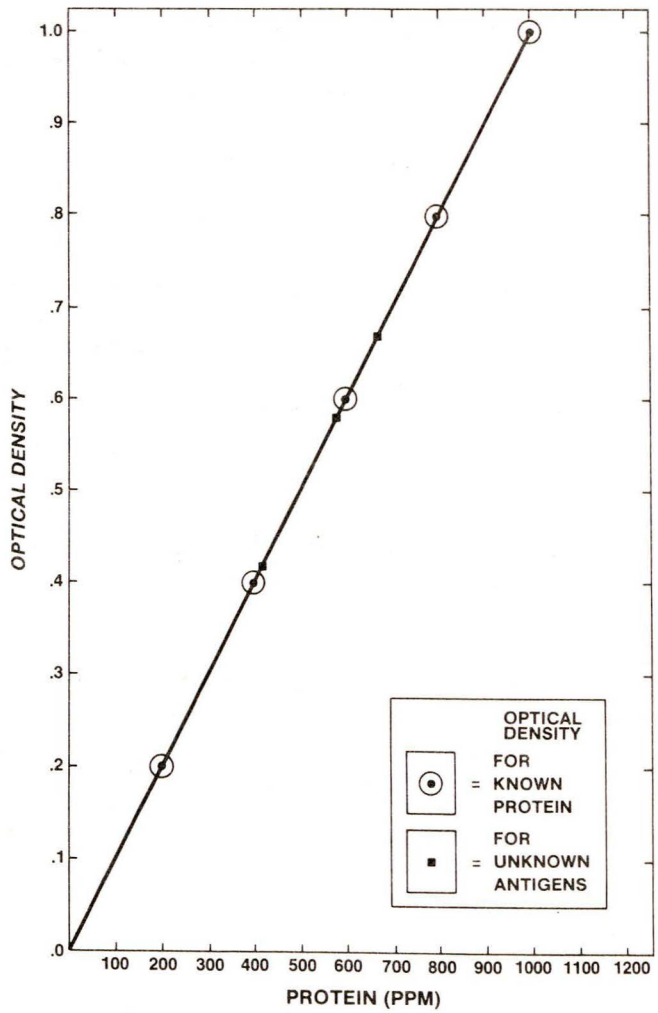


Figure 4.—*Determining protein concentration for assay*

Once the average is plotted, the parts per million of protein is achieved by reading across the standard protein graph to the regression line and down to the ppm numbers.

B. Because only 0.1 ml of antigen was used in this procedure (one-tenth concentration), multiply by 10 to determine the total concentration. This is the amount of protein in the sample.

   Example: In table 3–Sample A has an optical density of 0.42

   $$420 \times 10 = 4{,}200 \text{ ppm}$$

C. Bring all antigens to an equal concentration of 1,000 ppm. Because the desired ppm is 1,000, the ppm from sample A of 4,200 is 4.2 times as concentrated as needed. Therefore, the dilution factor equals 4.2 in this example.

$$\text{dilution factor} = \frac{\text{total concentration (ppm)}}{\text{desired concentration (ppm)}} = \frac{4{,}200}{1{,}000} = 4.2$$

D. To dilute to the appropriate concentration of 1,000 it is necessary to multiply the dilution factor by this amount of antigen. This is the final volume to be obtained.

   (total volume(ml) = dilution factor x desired volume of sample 1)
   = 4.2 x 10 ml
   = 42 ml

   Example: Vol. diluting agent = 42 ml total volume–10 ml antigen = 32 ml of diluting agent (0.9 percent saline solution)

   In this example, 32 ml of diluting agent is added to 10 ml of antigen that will be used to attain 1,000 ppm of antigen.

Diluting and Storing Antigen

Objective

To dilute the antigens obtained from liquid culture so they all have the same protein concentration. This is related to precipitation and intensity. Equalizing antigen proteins allows each antigen to react with the antibodies on an equal basis in the double diffusion plates.

Materials

1–2 ml pipette/sample
1–150 ml beaker/sample
1–250 ml beaker/sample for saline solution
1–250 ml graduated cylinder
0.9 percent physiological saline solution (see Appendix)

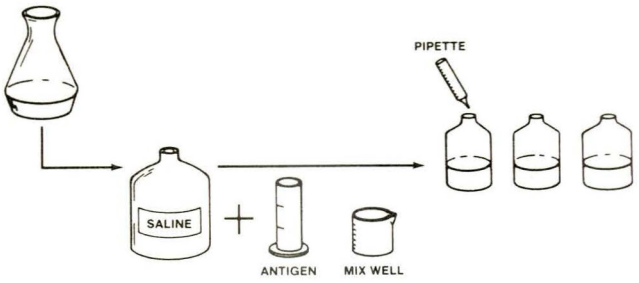


Figure 5.—*Diluting and storing antigen. (1) Place 10 ml antigen in a flask. (2) Add the required amount of chilled saline solution as determined from the dilution calculations and mix completely. (3) Pipette 2 ml diluted antigen into each half-dram serum vial, label, and freeze immediately.*

Ice and container
Half dram vials (8/sample)

Procedure

Using the previously calculated dilution factors, add the required amount of saline solution (diluting agent) to each antigen sample.

A. Put 10 ml of antigen in a beaker (fig. 5). Add the required amount of chilled saline solution as determined from the previous dilution calculations. Mix completely.
B. Pipette 2 ml of diluted antigen into each half-dram serum vial.
C. Label vials and freeze immediately.

Cross-Absorption of Antigens and Antibodies

Objective

Eliminate excess, non-characteristic proteins that will interfere with the 'monospecific'[3] diffusion test.

We have discovered that the antiserum prepared against the antigen for the European strain of *G. abietina* cross-reacts to some extent with the antigen for the North American strain. (Likewise for the North American antiserum and the European antigen.) It appears that some of the protein groups of the European strain are similar to those in the antigen for the North American strain. The presence of these common proteins when plated out in the double diffusion plates generates confusing precipitin bands that do not indicate identical strains of antigen to the antibodies, but rather a trace of *similar* proteins.

[3]*See glossary.*

The process of cross-absorption removes these unwanted proteins that are common to both strains using reciprocal absorption.

In our procedure two strains of *G. abietina* are involved. Antisera are prepared against each (as explained in Producing Antiserum Section). In the cross-absorption procedure for these two strains, two separate absorptions must be completed. North American antiserum is mixed with European antigen. The protein antibodies common to both strains react to form a precipitate that can be removed from the North American antiserum. This leaves only antibodies specific for the North American strain. The European antiserum is also mixed with the North American antigen. Again common protein antibodies are precipitated out leaving only antibodies that will react with proteins specific for the European strain.

To absorb all the common antibodies, and yet not dilute the serum more than necessary, the optimum proportions between the antiserum and the heterologous antigen must be determined. Through trial and error we have arrived at the following cross-absorption levels: 1 part North American antiserum + 2 parts European antigen; 1 part European antiserum + 1 part North American antigen.

Materials

Antiserum prepared against specific antigens
Antigens derived from fungus material
Centrifuge tubes–one for each antiserum
Centrifuge
Pipettes
Pipette dispenser

Procedure

Using the established levels of antiserum to antigen, combine both and mix gently in labeled centrifuge tubes (Fig. 6). Cover tubes with Parafilm (see Appendix), let stand upright in a test tube rack for 24 hours at room temperature. A noticeable precipitate forms that consists of the excess proteins not directly involved with the antibodies used in the analysis (Crowle 1961). Centrifuge the precipitate and pour off the supernatant, being careful not to resuspend any of the precipitate.

Now test the absorbed antiserum against further heterologous antigens as a check that absorption is complete, and against homologous antigens as a check for the presence of antibodies specific for the fungi being tested. (This test is incorporated in the double-diffusion plates—Ouchterlony Double Diffusion Method Section.)

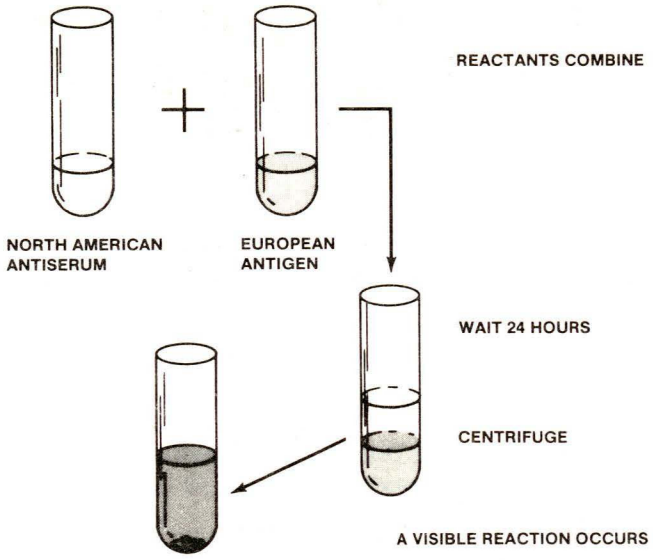


Figure 6.—*Cross-absorption of antigens to antiserum.*

Appendix

A. Source of known protein. Bovine Albumin (Fraction V) Eastman Kodak Co., Rochester, NY. 14650 Cat. #136 9750

B. Making the copper reagent–(for six or less samples).
   1. Combine the following three ingredients (in the specified amounts) and place in a repeating pipette that dispenses 5 ml.
      a. 250 ml of 2 percent Na_2C0_3 in 0.1N NaOH made by adding 20 g $NaC0_3$ and 4 g NaOH to 1 liter of distilled water.
      b. 2.5 ml of 2 percent $Na_2C_4H_4O_6$ (sodium tartrate) made by adding 20 g $Na_2C_4O_6$ to 1 liter of distilled water.
      c. 2.5 ml of 1 percent $CuS0_4$ (copper sulfate) made by adding 10 g $CuS0_4$ to 1 liter of distilled water.
   2. Mix all ingredients with a magnetic stirrer until dissolved.

C. Folin solution
   Pipette 0.5 ml phenol reagent (can be purchased from Fisher Scientific Co., Fair Lawn, New Jersey, under the name of Folin–Ciocalteau, solution 2n.)

D. Diluting and storing antigen
   Physiological saline solution–0.9 percent saline made by adding 9 g NaCl to 1 liter of distilled water.

PRODUCING ANTISERUM

Producing Antigens for Injecting Rabbits

Objective

To produce the required amount of antigen at 2,000 ppm protein level for injecting into rabbits to produce antibodies.

Materials

Fungus culture for producing antigen
24–500 ml Erlenmeyer flasks
Liquid growth media
Standard equipment for harvesting and homogenizing antigens
20 half-dram vials (sterilized)

Procedure

A. Using the procedure as outlined in the Handling the Test Organism and Producing Antigen Section (on growth in liquid culture), inoculate 24–500 ml Erlenmeyer flasks with the fungus culture that will be used to produce antibodies.
B. After approximately 21 days of growth in a 20°C dark incubator, harvest, homogenize, and filter the mycelial material as for regular antigen production as described in the Preparing Antigens for the Double Diffusion Test Section.
C. After determining the protein concentration, adjust the antigens to 2,000 ppm of protein (using the procedure outlined in the Preparing Antigens for the Double Diffusion Test Section).
D. Place antigen material in sterilized vials. Label and freeze until needed for injecting rabbits.

Injecting Antigens

Objective

Produce a homologous antiserum by injecting a complementary protein into a test animal.

Materials

2 New Zealand doe rabbits for each antiserum needed (see Appendix)
Freunds complete Bacto adjuvant (see Appendix)
Freunds incomplete adjuvant (see Appendix)
Soluble antigen
Hypodermic syringe–as specified by commercial lab
Needle–as specified by commercial lab

Procedure

Injecting fungus antigen into the rabbit stimulates the animals immune system to produce antibodies in direct response to the foreign protein. This antibody response is the basis of the serological system. After injecting antigens, the rabbit is checked periodically to determine the amount of antibody production (titer). This is done by a trial bleeding.

A. **Injection site information**—rabbit injection can be subcutaneous (under the skin), intraperitoneal (into the adbominal cavity), intravenous (into a vein), or intramuscular (into the muscle). The most effective method of antiserum production employs a combination of the listed injection sites.
B. **Adjuvant information**—an adjuvant is usually mixed with the antigen at the time of injection. This stimulates and prolongs the immune response and increases antibody production. Because of variation in antibody production among rabbits, it is best to use at least two rabbits for each strain of antiserum being produced.
C. **Laboratory information**—although injecting rabbits with antigens and the subsequent bleedings are not difficult procedures, many laboratories do not have the necessary facilities for keeping the test animals. Several commercial laboratories (see Appendix) will handle this process for the serologist. The normal procedure is for the serologist to supply the test antigen and the injection schedule. If animal facilities are not available, this is probably the most economical way to obtain the necessary serum.
D. **Injection schedule**—schedules may vary in accordance to the serologist's needs. Although many schedules have been adapted for producing antibodies, the goal is to obtain a high titer (see Appendix) in the final serum. This antibody titer consists of the highest dilution of serum that will give a reaction with an antigen. For our purposes a titer of 1:32 is extremely good–1:16 being the usual titer obtained. Anything lower than 1:8 is not acceptable.

Table 5 shows the injection schedule that has given the required titer in this study.

Harvesting and Storing Antiserum

Objective

Recover antibodies from the rabbit by obtaining blood and allowing it to clot, which makes it possible

Table 5.—*A sample injection schedule that has given the necessary titer*

Day	Injection site	Amount injected	Material injected
		ml	
1	Intramuscular	1	Freunds complete Bacto adjuvant–minus antigen
	Intramuscular	1	Soluble antigen–minus adjuvant
11	Subcutaneous	1	Freunds incomplete Bacto adjuvant
	Subcutaneous	1	Soluble antigen
21	Intraperitoneal	1	Freunds complete Bacto adjuvant
	Intraperitoneal	1	Soluble antigen
23		—Trial bleeding to check titer—	
			(no adjuvant added)
31	Intravenous	½	Soluble antigen
35	Intravenous	1	Soluble antigen
39	Intravenous	1	Soluble antigen
43	Intravenous	1	Soluble antigen
45		—Final bleeding for serum—	

for serum to be separated from the clotted cells. This fluid part of the blood (serum) contains the antibodies.

Procedure

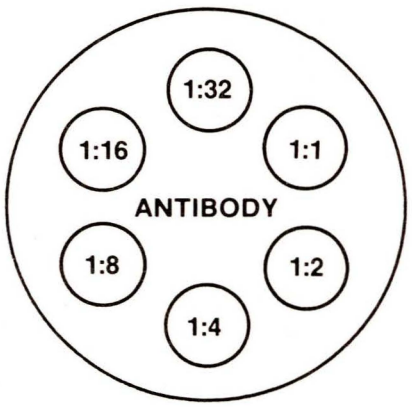
A. After extracting whole blood from the rabbit, let the blood sit in a covered beaker at room temperature for 1 to 2 hours until a clot forms.
B. Place the covered beaker at 4°C for 24 hours the clot will contract.
C. Decant the liquid serum from the beaker leaving the clot in the bottom. Save the clot. More serum can be extracted by letting the clot sit for an hour, then centifuging it for 10 minutes at 10,000 rpms.
D. Dispense the serum into vials, usually 1 to 2 ml per vial. Freeze vials immediately.

NOTE: Repeated freezing and thawing will impair the viability of the serum antibodies. If stored and sealed properly, the antiserum will remain viable for at least 12 months.

Appendix

A. Injecting antigens.
New Zealand rabbits–5 lbs.
Commercial laboratories–Green Hectares, 4583 Schneider Drive, Oregon, WI 53575.
Hypodermic syringe.
Freunds complete and incomplete adjuvant provided by the commercial lab.

B. Determining titer of antiserum.
1. Use about ½ ml of the antiserum (keep the rest frozen).
2. Label 6 vials–1:1, 1:2, 1:4, 1:8, 1:16, and 1:32.
3. Using a micropipette gun, dispense 100 µl antiserum to 100 µl 0.9 percent saline solution (1:1), 100 µl antiserum to 200 µl saline (1:2), 100 µl antiserum to 400 µl saline (1:4), 100 µl antiserum to 800 µl saline (1:8), 25 µl antiserum to 400 µl (1:16), and 25 µl antiserum to 800 ; gml saline (1:32).
4. Following dilution, mix the samples by inverting the vials.
5. Test the titer by making several agarose gel diffusion plates as follows.



6. Read the plates in about 48 hours. After inoculation, keep the plates at room temperature and do not move them.
7. The titer is the highest dilution with a visible white precipitation band between the center well and the dilution well.

C. Diluting and storing antigen
0.9 percent saline solution = 9 g NaCl/1 liter distilled water

D. Two dimensional Ouchterlony double diffusion plates.
1. Petri dishes–50 mm x 9 mm
Falcon, 1950 William Dr., Oxnard, CA 93030.
2. Agarose media
   a. Combine the following two materials: 1 g–agarose–J. T. Baker Chemical Co., Phillipsburg, NJ, and 100 ml 9 percent physiological saline.
   b. Place in 600 ml beaker–place in a boiling water bath.
   c. Wait until solution is clear.
   d. Add 0.05 g sodium azide–NaN_3 FW 65.0 Practical grade J. T. Baker.

OUCHTERLONY DOUBLE DIFFUSION METHOD

Diffusion Plates and Design

Objective

Determine and identify responses between homologous antigen and antibody reactants that produce a line of precipitation where common proteins diffuse and meet in the gel. This is the final procedure used to determine the strain of *G. abietina*.

Materials

Agarose–sodium azide media (see Appendix)
Design for gel diffusion plate
4 mm diameter agar punch/cork borer
Disposable pipettes and dispenser
5 cm diameter tight lid plastic petri dishes

Procedure

Cut a series of wells into the gel diffusion plates and fill with the unknown antigens and the cross-absorbed serum. As these materials diffuse out from the wells, precipitation bands form between the wells of the antigen and the antiserum containing common proteins. Interpretation of these bands identifies the strain of the unknown antigen. The design and use of the diffusion plates can vary as long as the following rules are followed:

A. An antigen well must be placed opposite an antibody well so the two solutions can diffuse toward each other and allow a precipitate band to form if the antigen-antibody reaction takes place.
B. All outer wells should be equal in size and distance from the center well.

Preparing Double Diffusion Plates

A. Place 6 ml of warm agarose in each petri plate. After the agarose has solidified, place a paper pattern under each petri dish and cut the agar wells using the agar punch. Well diameter is 4 mm. The distance between the center well and each outer well is also 4 mm. Remove the agar plug from the wells by using a micro-suction or a micro-harpoon, being careful not to damage the sides of the agar well or to lift the agar from the bottom of the plate because solutions could leak into other wells.
B. Store unused gel plates at 3 to 10°C in an airtight container that has moist paper towels at the bottom. (The added moisture at the bottom allows for a longer storage period.)

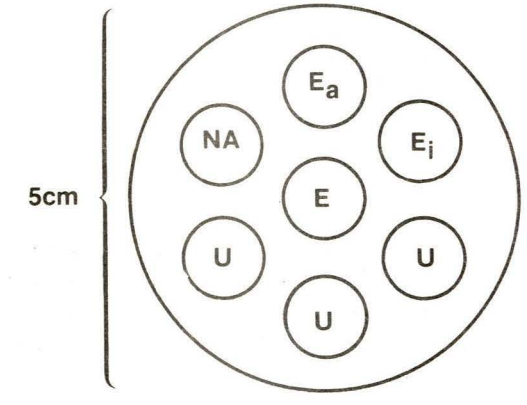
The Double Diffusion Plate Design

Figure 7 is an example of a pattern and labeling system for the gel diffusion plate used to determine the European strain of *G. abietina*.

Filling the Double Diffusion Plate Wells

When filling the plate wells, the following points should be remembered.

A. Fill the wells as quickly as possible because diffusion takes place rapidly.
B. Use at least three plates to determine each strain. This replication provides more accurate results.



E = known European antiserum (previously cross-absorbed);

E_a = known European antigen used to make antiserum E;

E_i = known European antigen different from E_a;

NA = known North American antigen; and

U = unknown antigens to be tested.

NOTE: Permanently mark the top and bottom of petri dish to identify positioning when the lid is removed.

Figure 7.—*Pattern and labeling system of the gel diffusion plate used to determine the European strains of* G. abietina.

C. Place equal amounts of antigen in each well for the best results. This is done by using micropipettes.
D. Avoid overflowing wells with antigen or antiserum, because this will give erratic readings.
E. Once the wells are filled, carefully replace the plate covers to avoid spilling the antigens and antiserum. Store in moist, tight containers at room temperature. Plates can be read in about 48 hours.

Interpreting Plates

Because cross-absorbed serum is used, the plate interpretation is simple. The petri dish offers a clear view of the precipitin bands.

Procedure

A. Approximately 48 hours after the wells are filled with antigens and antibody, diffusion will become visually complete. When plates are held over a light source, precipitin bands become distinguishable.
B. In the Ouchterlony method, the position of the precipitin bands indicates the antigen strain present in the well (fig. 8).

Analyzing Precipitin Bands

A. A *positive reaction* is indicated by a continuous precipitin band (fig. 8).
B. *Negative reactions* are indicated by precipitin bands that cross-over or have spurs. This may be a result of incomplete cross-absorption or an excess of antigen or antibody (figs. 9, 10).

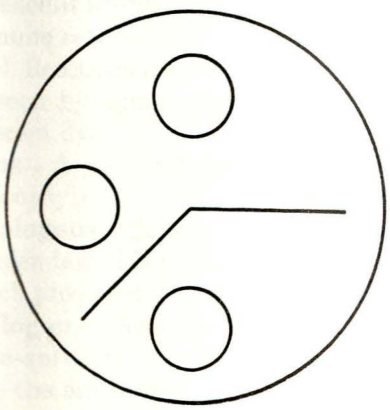


Figure 8.—*A continuous precipitin band indicates a positive reaction.*

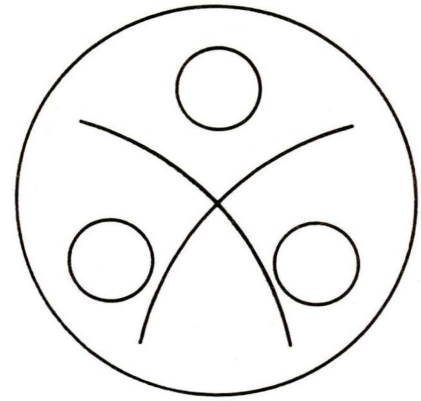


Figure 9.—*Precipitin bands that cross-over indicate a negative reaction between two distinct antigens that react with a distinct antibody in the antiserum.*

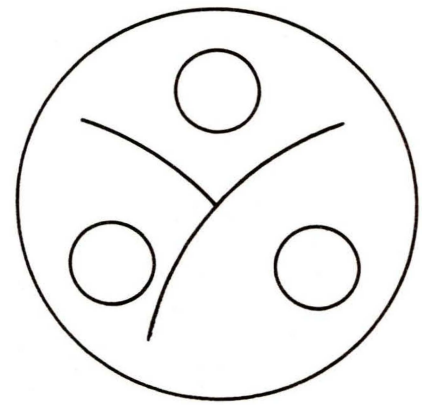


Figure 10.—*Precipitin bands that form a spur indicate a negative reaction between two distinct antigens that are related but heterologous.*

Some examples of diffusion reactions when testing for the European strain of *G. abietina* (fig. 11).

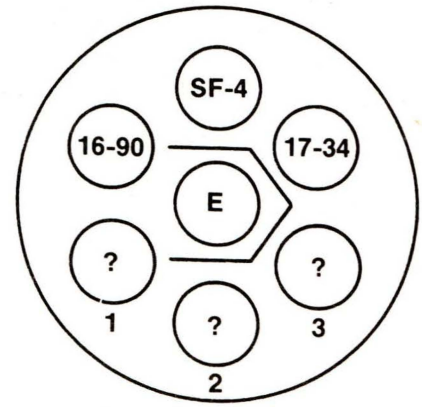


Figure 11a.—*All the antigens, except 16-90 and unknown #1 are reacting with the center wall. Because a visible precipitate is present at the interface of antibody and antigens of #2 and #3, the reaction indicates common proteins specific to the European strain of* G. abietina.

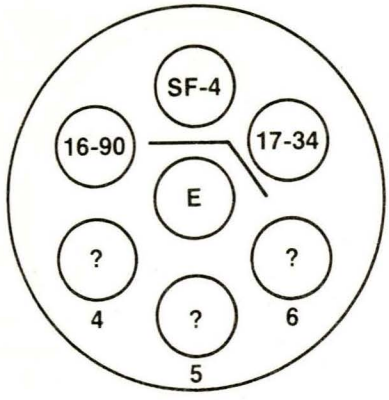


Figure 11b.—*None of the unknown antigens are reacting with the antibody. The only reactions occurring are between known homologous antigens. The unknowns in wells 4, 5, and 6 do not contain the protein specific to the European strain of* G. abietina.

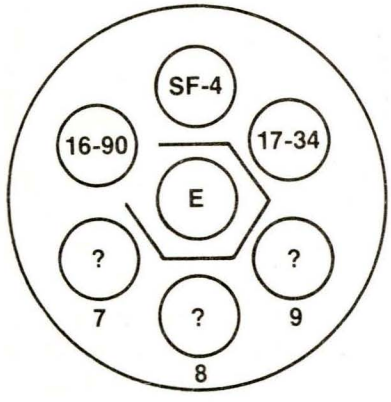


Figure 11c.—*All unknown antigens display a positive precipitate with the antibody, indicating common proteins. The known North American antigen 16-90 shows no reaction with the European antibody. The unknowns in wells 7, 8, and 9 contain the protein specific to the European strain of* G. abietina.

Some examples of diffusion reactions when testing for the North American strain of *G. abietina* (fig. 12).

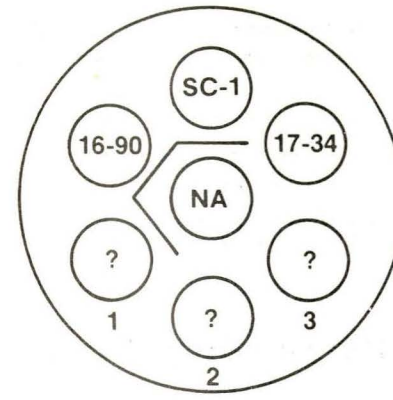


Figure 12a.—*Unknown antigen #1 is the only unknown antigen (other than the known homologous antigens) that is reacting with the antibody. The precipitan band at the interface of antigen and antibody indicates proteins specific to the North American strain of* G. abietina.

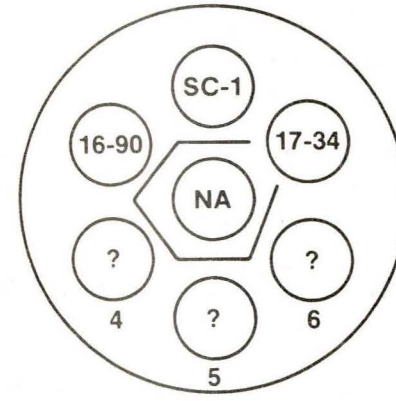


Figure 12b.—*All of the unknown antigens are reacting with the antibody and so are the known antigens except for the European strain. Wells 4, 5, and 6, therefore contain the protein specific for the North American strain of* G. abietina.

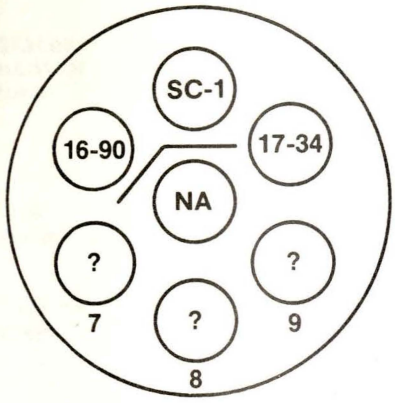


Figure 12c.—*None of the unknown antigens display a positive precipitate with the antibody, which indicates no common proteins specific to the North American strain of* G. abietina *in the double diffusion plate.*

GLOSSARY

Adjuvant—Substance added to antigens prior to injecting into a test animal to enhance activating properties and stimulate antibody production.

Antibody—A specific protein V-Globulin produced by an animal in direct response to the introduction of an antigen.

Antigen—A foreign protein that stimulates the production of specific antibodies when introduced into an animal.

Antiserum—Serum that contains antibodies.

Conidia—Small, asexual spores produced by fungi.

Cross-absorption—The process by which a portion of antibodies in an antiserum complex are precipitated out leaving only proteins specific to one strain.

Fluorescent immuno-assay—Procedure by which immune reactions can be observed at the cellular level. Reactions between antigen and antibody can be seen by using antibody conjugated to a fluorescent dye.

Fungus—A multicellular thallophyte that lacks chlorophyll.

Heterologous—Serological reactions in which the antigen tested is related but not the same as that which produced the antibody.

Homologous—Reaction that is identical to the antigen-antibody reaction of the antigen used to produce the antibodies.

Immunodiffusion—The separation of an antigen complex into discrete parts through difference in ability to pass through media.

Immunoelectrophoresis—A method using electrically charged particles to separate proteins in serum. Protein antigens are spread to specific areas along a line.

Immunology—Science of the immune response in animals.

Monospecific—Specific to only one antibody complex.

Mycelium—A mass of fungus filaments.

Ouchterlony Double Diffusion—The diffusion of both antigens and antibodies in a solid-liquid gel to form a precipitate at the interface.

Pycnidia—The fruiting bodies of certain fungi.

Proteins—Organic compounds of high molecular weight that contain nitrogen and yield amino acids on hydrolysis.

Serology—A branch of science that deals with the study of antigen-antibody reactions.

Serum—Cell-free and fibrin-free fluid expressed from clotted blood.

Titer—The strength of a solution as established through titration.

Titration—The measuring of how much of another substance it is necessary to add to a solution in order to produce a given reaction.

LITERATURE CITED

Ball, E. M.; Aapola, A. I. E.; Duffus, J. E.; Hamilton, R. I.; Ragetli, H. W. J.; Rochow, W. F.; Shepard, J. F. Serological tests for the identification of plant viruses. American Phytopathological Society Plant Virology Committee; 1974. 31 p.

Crowle, Alfred J. Immunodiffusion. Chapter IV, Immunodiffusion. New York: Academic Press; 1961.

Dorworth, C. E.; Krywienczyk, J. Comparisons among isolates of *Gremmeniella abietina* by means of growth rate, conidia measurements, and immunogenic reaction. Can. J. Bot. 53: 2506-2525; 1975.

Dorworth, C. E.; Krywienczyk, J.; Skilling, D. D. New York isolates of *Gremmeniella abietina* (*Scleroderris lagerbergii*) identical in immunogenic reaction to European isolates. Plant Dis. Rep. 61: 887-890; 1977.

Lowry, O. H.; Rosbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275; 1951.

Skilling, Darroll D.; Kienzler, Mariann.
A serological procedure for identifying strains of *Gremmeniella abietina*. Gen. Tech. Rep. NC-87. St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station; 1983. 15 p.

This manual gives detailed laboratory serology procedures necessary to determine the identity of isolates of *Gremmeniella abietina* by the gel double diffusion method. The process is described from the arrival of the field sample to the reading of the precipitin bands on the diffusion plate.

KEY WORDS: Antigens, antibodies, double diffusion, mycelial cultures, titer, precipitin bands.

U.S. GOVERNMENT PRINTING OFFICE:1983-765-129/5103